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ORIGINAL ARTICLE

Development and Evaluation of a Loop-mediated Isothermal Amplification (LAMP) Assay for Rapid Detection of Chinese Giant Salamander Ranavirus

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Abstract Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid diagnostic method that can amplify rapidly a target template under isothermal conditions. In this study, a LAMP assay for rapid detection of Chinese giant salamander ranavirus (CGSRV) was developed from culture isolates and clinical samples. The LAMP assay was developed by designing one set of four specific primers, targeting the major capsid protein (MCP) gene of CGSRV. Reaction time and temperature were optimal for 40 min at 62°C. The developed LAMP assay is specific and highly sensitive for CGSRV detection, the detection limit could reach about 5 copies of cloned viral genomic fragments. The sensitivity of the LAMP assay was about 1000 and 10-fold higher than that of both conventional and nested PCR, respectively. The LAMP amplification produces a typical ladder-like pattern of products on an agarose gel that can be visually inspected after addition of ethidium bromide. The LAMP assay was evaluated further with clinical samples, and the results indicated the suitability and simplicity of the test as a rapid diagnostic tool for the detection of CGSRV.

Keywords CGSRV, Loop-mediated isothermal amplification (LAMP), ranavirus, Chinese giant salamander

1. Introduction

The Chinese giant salamander (*Andrias davidianus* Blanchard, 1871) is the world's largest amphibian species and it is classified as critically endangered by the International Union for Conservation of Nature and Natural Resources. Because of its biological, medical, and nutritional value, it has been cultured widely in many provinces of China for more than 30 years (Zhang *et al.*, 2002). Though recent improvements in techniques and instruments have allowed the farmers to expand their farming scopes, such conditions often provide enhanced disease susceptibility. Since 2008 a novel infectious disease called 'limbs swelling disease or ulcer disease' has been reported to cause high mortality (>40%) in cultured Chinese giant salamanders leading to potential economic impacts on China's farming (Dong *et al.*, 2011; Geng *et al.*, 2010; Geng *et al.*, 2011). Chinese giant salamander ranavirus (CGSRV) isolated from Chinese giant salamanders in 2010 has been confirmed to be associated with 'limbs swelling disease or ulcer disease'. CGSRV exhibits a typical icosahedron shape, with a diameter of 130–180 nm. Additionally, it can be propagated in *epithelioma papulosum cyprini* (EPC) cells and is known to cause an obvious cytopathogenic effect (CPE) at 20–25°C (Geng *et al.*, 2011; Zhou *et al.*, 2013). Comparison of CGSRV with other *Ranavirus* species, including FV3, KRV-1, soft-shelled turtle iridovirus (STIV) and *Rana grylio* iridovirus, revealed the high homology (95%–100%) of the nucleotide and amino acid sequences among major caspid protein (MCP), suggesting that CGSRV could be classified as a member of genus

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Ranavirus in the family of *Iridoviridae* (Geng *et al.*, 2011; Zhou *et al.*, 2013).

Several methods have been developed to detect CGSRV in Chinese giant salamanders including, cell culture (Geng *et al.*, 2011), electron microscopy (Geng *et al.*, 2010), conventional PCR (Zhou *et al.*, 2012b) and quantitative real-time PCR (Zhou *et al.*, 2012a). Among these methods, quantitative real-time PCR is effective in detecting the virus prior to the onset of infection. However, this method requires expensive equipment and reagents, thus limiting its use on a wide-scale basis. Conventional PCR, although affordable, has some intrinsic disadvantages such as the requirement for rapid thermal cycling, limited specificity and low amplification efficiency. Histopathological and electron microscopic methods used for virus detection often fails during the early stages of infection (Geng *et al.*, 2010; Geng *et al.*, 2011). Thus a simple, rapid and sensitive method needs to be developed towards early detection of CGSRV.

In order to overcome the limitations of conventional viral detection methods, a novel nucleic acid amplification method called loop-mediated isothermal amplification (LAMP), was developed by Notomi (Notomi *et al.*, 2000). The same method has been applied successfully for iridovirus diagnosis, including Singapore grouper iridovirus (SGIV; Mao *et al.*, 2008), turbot reddish body iridovirus (Zhang *et al.*, 2009), lymphocystis disease virus (LCDV; Li *et al.*, 2010), infectious spleen and kidney necrosis virus (ISKNV; Ding *et al.*, 2010), and soft-shelled turtle iridovirus(STIV; Liu *et al.*, 2011). The aims of the present study were to develop a LAMP based assay, for improved detection of CGSRV infection, and to evaluate the effectiveness of this tool for identification of CGSRV in Chinese giant salamanders.

2. Materials and Methods

2.1 Preparation of virus and plasmid DNA as LAMP template The CGSRV (isolated, identificated and stored in our laborary) suspension was inoculated onto the monolayer of EPC cells (a generous gift from the Pearl River Fisheries Research Institute in China) and the inoculated cells were incubated at 25°C in culture medium M199, supplemented with 2% fetal calf serum. The infected cells were collected after 24 h by centrifugation at 3000g for 30 min. The viral DNA extraction and purification was performed using the Takara universal genomic DNA extraction kit, version 3.0 (Takara, Dalian, China). The 470bp target sequence in the major capsid protein (MCP) gene (HQ684746) of CGSRV was amplified using PCR primers (P1: 5′-ATGCGGATAATGTTGTGGT-3′ and P2: 5′- TTCTCACACGCAGTCAAGG -3′). The PCR products were separated and purified according to the instructions of TIANgel Midi Purification Kit (DP209) (Takara, Dalian, China). The PCR products were then cloned into pMD-19T Vector to construct recombinant plasmid, and trans-formed into the DH5α competent cells. The cells were pre-cultured, and transformed cells were then subjected to large-scale culture for plasmid DNA extraction and purification. These plasmids were purified using the Qiagen kit (Sangon, Shanghai, China) for plasmid purification. The recombinant plasmid DNA was stored at –20°C for further analysis.

2.2 Design of primers for LAMP The CGSRV specific primers for the LAMP assay were designed according to the MCP gene sequence of the CGSRV (GenBank accession no. HQ684746), using primer explorer software version 4 (https://primerexplorer.jp/e/). A set of four primers, consisting of two outer and two inner primers, was designed as shown in Table 1. The forward inner primer (FIP), consisted of the complementary sequence of F1C (21nt) and F2 (18nt). The backward inner primer (BIP) consisted of B1C (22nt) and the complementary sequence of B2 (20nt). The outer primers consisted of F3 (18nt) and the complementary sequence of B3 (17nt). The FIP and BIP structure “the loop” throughout the reaction, while F3 and B3 facilitate initial strand displacement during early DNA synthesis.

2.3 Optimization of CGSRV-LAMP reaction conditions LAMP reactions were conducted as described by Notomi *et al.*(2000).The LAMP assay was conducted in a 25-μl reaction mixture containing 1 μl of each primers (F3 (10 μM), (B3 (10 μM), (FIP (50 μM), (BIP (50 μM), 0.6 μl of 1 U uracil-DNA glycosylase (Shinegene, Shanghai, China), 3.5 μl of dNTPs (25 mM), 4 μl of 10×Thermopol buffer, 1 μl (eight units) of Bst polymerase (New England Biolabs Inc., Beverly, MA, USA), 1.0 μl betaine (100 mM), 3 μl $MgCl_2$ (25 mM), and 2 μl of template (the recombinant plasmids) and 5.9 μl ddH2O. For determining the optimal reaction temperature, the reaction mixture was incubated in a heating block at 60, 61, 62, 63 and 64°C for 60 min, respectively, and then heated at 80°C for 2 min to terminate the reaction. To determine the optimum time for amplification, the LAMP reaction was performed at 62°C for different time periods at 10, 20, 30, 40, 50, 60 and 90 min. The LAMP products were electrophoresed on a 2.0% agarose gel, stained and visualized by a gel imaging system.

2.4 Specificity of the CGSRV-LAMP assay The specificity of CGSRV-LAMP assay was evaluated by using the templates from various viruses and bacteria including infectious spleen and kidney necrosis virus (ISKNV), marbled sleepy goby iridovirus (MSGIV), koi herpesvirus (KHV), Cyprinid herpesvirus II (CyHV-2), *Edwardsiella tarda*, *Aeromonas hydrophila*, and *Aeromonas veronii*. DNA from non-infected Chinese giant salamander was used as a negative control to determine any non-specific amplification.

2.5 Sensitivity of the CGSRV-LAMP assay To assess the sensitivity of CGSRV-LAMP, the recombinant plasmids containing the PCR product of CGSRV (constructed above) were estimated by ND-1000 ultraviolet spectrophotometer (Nano-Drop, Wilmington, Delaware, USA) and amplified in a serial of 10-fold dilution by using the optimum reaction system and conditions for LAMP determined above, and compared with both conventional and nested PCR detection method established in reported literature Liu *et al.*(2014) and Zhou *et al.*(2012b).

2.6 Visualization and detection of the LAMP products Ethidium Bromide (EtBr) solution (10 ug/ml), at 1:100 concentration was used for visual inspection of the LAMP reaction. The reaction was considered positive, if the addition of EtBr changed the solution color from orange to pink, thereby indicating the presence of LAMP products. In contrast, if the color remains orange, the reaction was considered negative indicating that LAMP products were not generated.

2.7 Application of CGSRV-LAMP assay for clinical diagnosis The suitability of the assay for detection of CGSRV was evaluated by comparing the detection results of the LAMP assay for 95 Chinese giant salamander samples against both conventional and nested PCR. The generated products were analyzed by EtBr stain, as described above.

3. Results

3.1 Optimization of CGSRV-LAMP reaction conditions The LAMP reaction was carried out using CGSRV DNA as a template to determine the optimal temperature and reaction time. The LAMP products appeared as a ladder-like pattern (with many bands of different sizes) at 60, 61, 62, 63, and 64°C (Figure 1). Although the amplification patterns at these different temperatures were quite similar, the band clarity was most prominent at 62°C. Therefore, 62°C was considered as the optimal temperature and was used in the subsequent experiments. In addition, optimal conditions for reaction time of LAMP assay were determined by performing reaction for 10, 20, 30, 40, 50, 60, and 90 min, respectively. These results indicated that no amplification of product was present, when reaction time was less than 30 min (Figure 2). The clarity of specific bands was similar for reaction times of 40, 50, 60, and 90 min. Hence, the optimized condition chosen was 62°C for 40 min.

3.2 Specificity of the CGSRV-LAMP assay To confirm specificity of amplification, LAMP reactions were analyzed against the templates obtained from CGSRV and

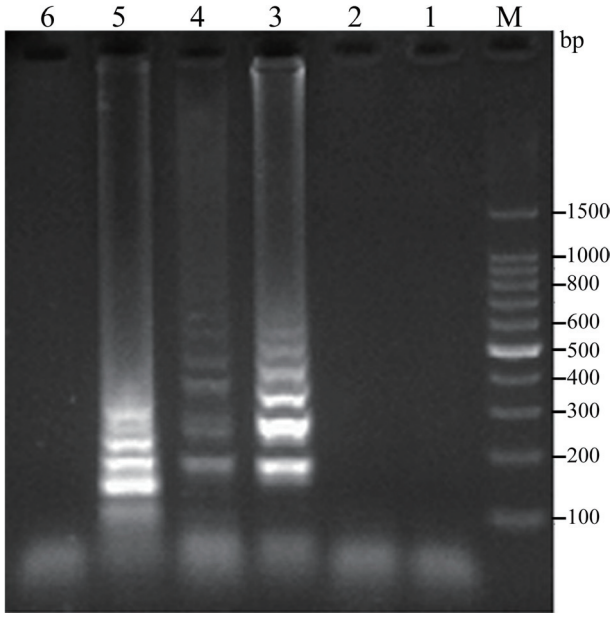

Figure 1 Determination of the optimal temperature for the LAMP assay by agarose gel electrophoresis. Lane M: 1500 bp DNA ladder; Lane 1-5: LAMP reactions at 60, 61, 62, 63, and 64°C, respectively; Lane 6: negative control.

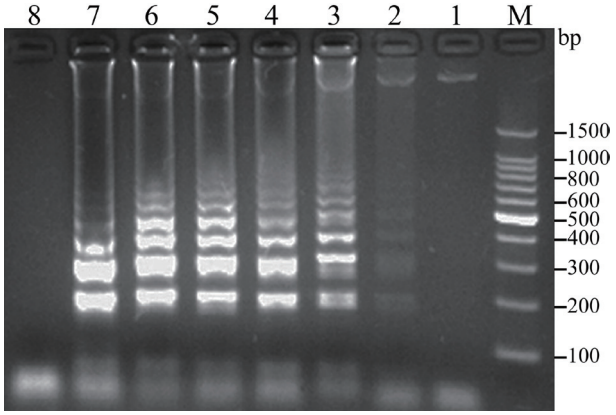

Figure 2 Determination of amplification time for the LAMP assay. Lane M: 1500 DNA ladder; Lane 1-7: LAMP reactions at 10, 20, 30, 40, 50, 60 and 90 min, respectively; Lane 8: negative control.

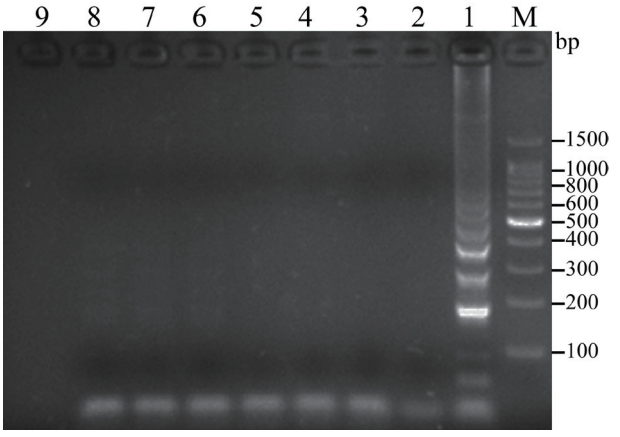

Figure 3 Determination of the specificity of the LAMP assay by agarosegel electrophoresis. Lane M: 1500 DNA ladder; Lane 1: CGSV, Lane 2-8: koi herpesvirus (KHV), Cyprinid herpesvirus II (CyHV-2), marbled sleepy goby iridovirus (MSGIV), infectious spleen and kidney necrosis virus (ISKNV), Edwardsiella tarda, Aeromonashydrophila, and Aeromonas veronii. Lane 9: negative control.

seven other aquatic animal viruses and bacteria. As shown in Figure 3, LAMP amplifications could only be detected from CGSRV, and not from other viruses and bacteria. Taken together, these results confirm that the LAMP assay is highly specific to CGSRV.

3.3 Sensitivity of the CGSRV-LAMP assay The sensitivity of the detection limits for the CGSRV-LAMP assay was evaluated in comparison to PCR, and nested PCR. These reactions were carried out using the 10-fold serial dilution of CGSRV DNA. The initial amount of input DNA was 250 μg (5×10^6 copies/μl). These results indicated that LAMP was able to detect the 10^{-6} diluted template corresponding to about 5 copies of cloned viral genomic fragments (Figure 4), which was 1000 and 10 times more sensitive than conventional PCR and nested PCR, respectively, suggesting that the LAMP assay was more sensitive than both conventional and nested PCR.

3.4 Visualization and detection of the LAMP products As the LAMP reaction progresses, the reaction by-products bind to magnesium ions and form a white precipitate of magnesium pyrophosphate. Positive reactions can then be identified by the production of a white precipitate. Color changes were noted on visual inspection of LAMP reaction tubes after the addition of EtBr: positive samples turned pink, while negative samples and no template control reactions remained orange (Figure 5). These results are in agreement with gel electrophoretic observations.

3.5 Application of CGSRV-LAMP assay for clinical diagnosis The LAMP, nested PCR and conventional PCR methods were carried out for detection of CGSRV from 95 Chinese giant salamander samples. All 55 infected samples were positive for the presence of CGSRV by LAMP, while none of the eight healthy samples demonstrated the presence of CGSRV. Similar results were obtained using nested PCR and conventional PCR. In addition, the LAMP assay could successfully detect CGSRV in 30 Chinese giant salamanders, from 32 asymptomatic suspicious samples, however, in contrast, nested PCR and conventional PCR could only detect CGSRV in 28 and 22 of the samples, respectively (Table

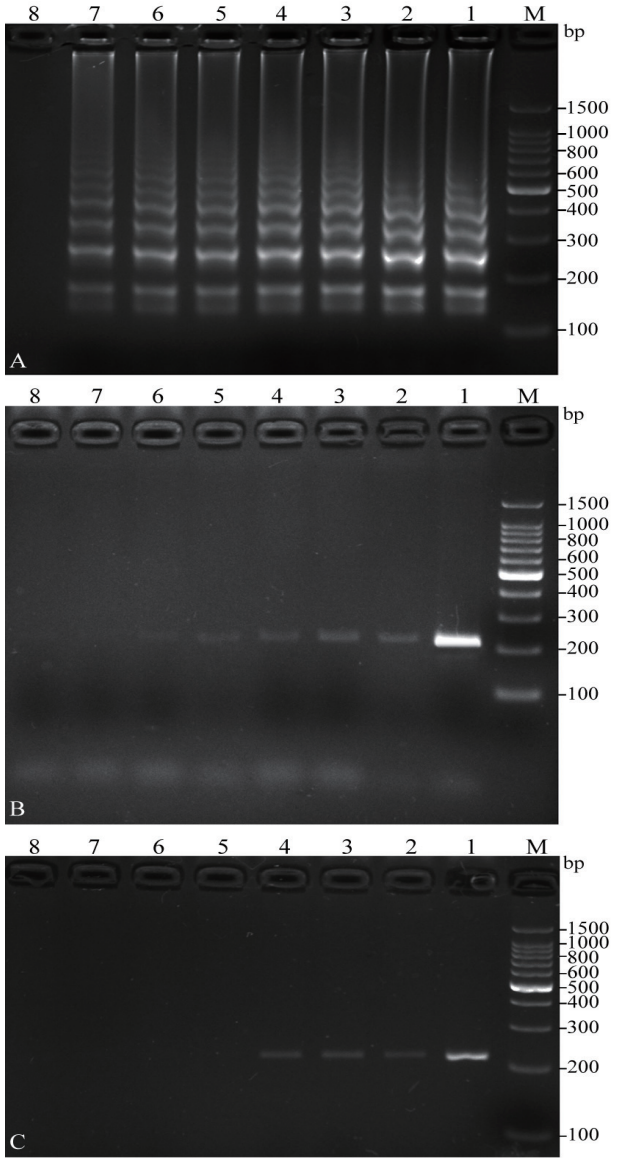

Figure 4 Determination of the sensitivity of LAMP (A), Nested PCR (B) and conventional PCR (C) using 10-fold serial dilutions of CGSRV DNA as template. Lane M: 1500 bp DNA ladder; lanes 1,2,3,4,5,6 and 7, the copies number of Pmd-19T-CGSRV was about 5×106, 5×105, 5×104, 5×103, 5×102, 50, and 5; lane 8: negative/blank control.

Table 1 Primers used for Loop-mediated Isothermal Amplification (LAMP) of CGSV.

primer	Nucleotide Sequence(5'-3')	Length	Gene position
F3	GAGGGCGTACTTTTGGGC	18	40-57
B3	ATGCCACCTCCATCCCA	17	229-245
FIP(F1C+F2)	TAACGTCACCCTGTCCGCTGA - CAGAGTTGTCACCTCCGC	39	109-129; 69-86
BIP(B1C+B2)	TCTGCCGTAATTGGTGGATCCG - GGCACCACCTCTACTCCTA	41	148-169; 200-218

Table 2 Detection of CGSV by LAMP, nested PCR and conventional PCR from infected, unknown and healthy samples.

Sample type	Collection place	Sample number	Results		
			Conventional PCR (positive/negative)	Nested PCR (positive/negative)	LAMP (positive/negative)
Infected	Sichuan, Ganshu	55	55/0	55/0	55/0
Unknown	Shanxi, Sichuan	32	22/10	28/4	30/2
Healthy	Sichuan	8	0/8	0/8	0/8

2). These data indicated that the detection sensitivity of LAMP was higher than that of both nested and conventional PCR, suggesting that LAMP can be used for the early diagnosis of CGSRV infection from clinical samples.

4. Discussion

Viruses in the genus *Ranavirus* are emerging killers with a wide host range, including fish, amphibians, and reptiles (Miller *et al.*, 2011; Whittington *et al.*, 2010; Williams *et al.*, 2005). It has been shown previously that members of the genus *Ranavirus* are potentially responsible for mass mortality of amphibians (Chinchar, 2002; Gray *et al.*, 2009; Robert, 2010). CGSRV, a novel member of the genus *Ranavirus*, poses a serious threat to Chinese giant salamander culturing and could cause substantial financial losses (Dong *et al.*, 2011; Geng *et al.*, 2011; Zhou *et al.*, 2013). Therefore, establishing a simple, rapid and reliable diagnostic method for CGSRV is of utmost importance and a key element to prevent further spreading of the disease. Currently available CGSRV diagnostic tests include histopathology, viral isolation, electron microscopy, conventional PCR and real-time PCR. Although PCR has been highly useful for detecting CGSRV, the technology is still technically demanding and requires more than 4–6 h to complete. In contrast, the LAMP reaction is performed under isothermal conditions and is relatively inexpensive. In addition, the LAMP method can amplify a few copies of DNA in less than 1 h (Mori *et al.*, 2001). The high amplification efficiency of the LAMP method is attributed to saving time of thermal change due to its isothermal reaction(Kalinina *et al.*, 1997). In this study, a specific and highly sensitive LAMP assay was developed for the rapid detection of CGSRV. The detection limit of LAMP is approximately 1,000 and 10 times more sensitive than conventional PCR and nested PCR.

In this study, reaction time and temperature of LAMP for CGSRV were optimized for 40 min at 62°C. The high sensitivity of the LAMP system makes it susceptible to false-positive results, due to carry-over or cross-contamination, therefore amplification and detection should be carried out in separate working areas. In addition, uracil-DNA glycosylase was added to the PCR reactions to control carry-over contamination which can lead to false-positive results. Furthermore, the specificity of amplification was thoroughly validated, to ensure that the primers specifically amplify the target sequence of the pathogen. To assess specificity of the LAMP assay, the templates from CGSRV and other aquatic animal viruses and bacteria were tested. The results confirm that the LAMP assay is highly specific to CGSRV. The LAMP assay was evaluated further with clinical samples, and the results indicated the suitability and simplicity of the test as a rapid diagnostic tool for the detection of CGSRV.

Use of nucleic acid stains allow a quick visualization of LAMP products, even without performing gel electrophoresis. In this study, the EtBr staining was performed for visual inspections of LAMP products. As expected, the color of a positive reaction of LAMP, turned to pink after the addition of EtBr, whereas the original orange color did not change, if a reaction product was not generated. Similarly, SRBY Green I is considered another useful nucleic stain; after addition of SRBY Green I, the positive LAMP products turned green, while the negative reaction products remained orange(Li *et al.*, 2010; Liu *et al.*, 2011; Soliman and Eimatbouli, 2006). However, in the system described in the present study, SRBY Green I nucleic staining failed to produce any visible colour changes. Further evaluations are required to explain why this phenomenon was observed.

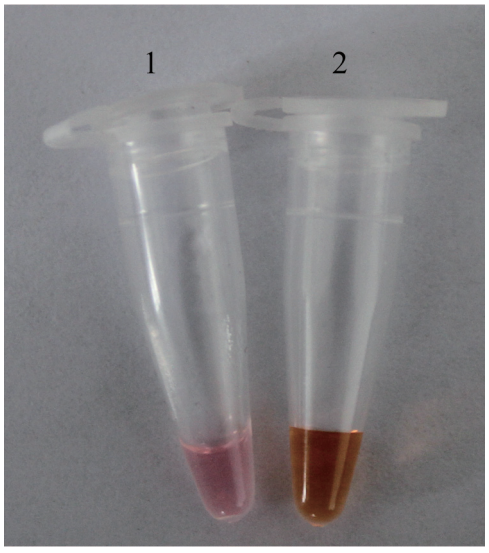

Figure 5 Visual observation for detection of LAMP products after EtBr staining. 1: positive CGSV reaction showing pink color; 2: negative CGSV reaction showing orange color.

A significant risk associated with SCGV infection is potential widespread outbreaks of limbs swelling disease or ulcer disease in Chinese giant salamanders. Thus, early detection of possible outbreaks can be achieved through enhance disease monitoring. This study reports the development of a LAMP assay which is an extremely rapid, specific, sensitive and convenient method to detect CGSRV DNA. Furthermore, this method requires simple reaction conditions and less time to obtain a result as compared to the traditional gel electrophoresis method. Therefore, this LAMP assay can potentially be used under field conditions, for rapid diagnosis of CGSRV, which would allow emergency control measures to be implemented quickly, to prevent further spread of the disease.

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